

1258649

TO AND TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

December 09, 2004

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM
THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK
OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT
APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A
FILING DATE.

APPLICATION NUMBER: 60/574,252
FILING DATE: *May 25, 2004*
RELATED PCT APPLICATION NUMBER: PCT/US04/37220

Certified by



Jon W Dudas

Acting Under Secretary of Commerce
for Intellectual Property
and Acting Director of the U.S.
Patent and Trademark Office



BEST AVAILABLE COPY

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. **ET311327599US**22151 U.S. PTO
601574252

052504

INVENTOR(S)

Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
STEVEN J.	SAUL	ARLINGTON, MA

Additional inventors are being named on the **SECOND** separately numbered sheets attached hereto**TITLE OF THE INVENTION (500 characters max)****Inhibition Assay Method and Device for Detection of Antibiotics**Direct all correspondence to: **CORRESPONDENCE ADDRESS** Customer Number:**000043211****OR**

<input type="checkbox"/>	Firm or Individual Name	
Address		
Address		
City	State	Zip
Country	Telephone	Fax

ENCLOSED APPLICATION PARTS (check all that apply)

- | | | |
|---|-----------|---|
| <input checked="" type="checkbox"/> Specification Number of Pages | 27 | <input type="checkbox"/> CD(s), Number _____ |
| <input checked="" type="checkbox"/> Drawing(s) Number of Sheets | 2 | <input checked="" type="checkbox"/> Other (specify) Express Mail Cert. |
| Fee transmittal; Check; Postcard | | |
| <input type="checkbox"/> Application Data Sheet. See 37 CFR 1.76. | | |

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

- | | |
|---|-----------------------------------|
| <input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. | FILING FEE
Amount (\$) |
| <input checked="" type="checkbox"/> A check or money order is enclosed to cover the filing fees. | |
| <input type="checkbox"/> The Director is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: _____ | |
| <input type="checkbox"/> Payment by credit card. Form PTO-2038 is attached. | |

\$80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

 No. Yes, the name of the U.S. Government agency and the Government contract number are: _____

[Page 1 of 2]

Respectfully submitted,

SIGNATURE TYPED or PRINTED NAME **RICHARD J. LONG**TELEPHONE **978-687-9200 X130**Date **MAY 25, 2004**REGISTRATION NO. **48252**

(if appropriate)

Docket Number. **0656-032-US2**

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

FEE TRANSMITTAL

for FY 2004

Effective 10/01/2003. Patent fees are subject to annual revision.

 Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT (\$ 80.00)

Complete If Known

Application Number	
Filing Date	MAY 25, 2004
First Named Inventor	STEVEN J. SAUL
Examiner Name	
Art Unit	
Attorney Docket No.	0656-032-US2

METHOD OF PAYMENT (check all that apply)

 Check Credit card Money Order Other None
 Deposit Account:

Deposit Account Number **50-3152**

Deposit Account Name **CHARM SCIENCES, INC.**

The Director is authorized to: (check all that apply)

- Charge fee(s) indicated below Credit any overpayments
- Charge any additional fee(s) or any underpayment of fee(s)
- Charge fee(s) indicated below, except for the filing fee to the above-identified deposit account.

FEE CALCULATION

1. BASIC FILING FEE

Large Entity	Small Entity	Fee Code (\$)	Fee Code (\$)	Fee Description	Fee Paid
1001 770	2001 385	Utility filing fee			
1002 340	2002 170	Design filing fee			
1003 530	2003 265	Plant filing fee			
1004 770	2004 385	Reissue filing fee			
1005 160	2005 80	Provisional filing fee	80		
SUBTOTAL (1) (\$)		80			

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

Total Claims	Independent Claims	Multiple Dependent	Extra Claims	Fee from below	Fee Paid
			-20** =	X	=
			-3** =	X	=

Large Entity	Small Entity	Fee Description	Fee Paid
1202 18	2202 9	Claims in excess of 20	
1201 86	2201 43	Independent claims in excess of 3	
1203 290	2203 145	Multiple dependent claim, if not paid	
1204 86	2204 43	** Reissue independent claims over original patent	
1205 18	2205 9	** Reissue claims in excess of 20 and over original patent	
SUBTOTAL (2) (\$)			

**or number previously paid, if greater; For Reissues, see above

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Small Entity

Fee Code (\$)	Fee Code (\$)	Fee Description	Fee Paid
1051 130	2051 65	Surcharge - late filing fee or oath	
1052 50	2052 25	Surcharge - late provisional filing fee or cover sheet	
1053 130	1053 130	Non-English specification	
1812 2,520	1812 2,520	For filing a request for ex parte reexamination	
1804 920*	1804 920*	Requesting publication of SIR prior to Examiner action	
1805 1,840*	1805 1,840*	Requesting publication of SIR after Examiner action	
1251 110	2251 55	Extension for reply within first month	
1252 420	2252 210	Extension for reply within second month	
1253 950	2253 475	Extension for reply within third month	
1254 1,480	2254 740	Extension for reply within fourth month	
1255 2,010	2255 1,005	Extension for reply within fifth month	
1401 330	2401 165	Notice of Appeal	
1402 330	2402 165	Filing a brief in support of an appeal	
1403 290	2403 145	Request for oral hearing	
1451 1,510	1451 1,510	Petition to institute a public use proceeding	
1452 110	2452 55	Petition to revive - unavoidable	
1453 1,330	2453 665	Petition to revive - unintentional	
1501 1,330	2501 665	Utility issue fee (or reissue)	
1502 480	2502 240	Design issue fee	
1503 640	2503 320	Plant issue fee	
1460 130	1460 130	Petitions to the Commissioner	
1807 50	1807 50	Processing fee under 37 CFR 1.17(q)	
1806 180	1806 180	Submission of Information Disclosure Stmt	
8021 40	8021 40	Recording each patent assignment per property (times number of properties)	
1809 770	2809 385	Filing a submission after final rejection (37 CFR 1.129(a))	
1810 770	2810 385	For each additional invention to be examined (37 CFR 1.129(b))	
1801 770	2801 385	Request for Continued Examination (RCE)	
1802 900	1802 900	Request for expedited examination of a design application	
Other fee (specify)			
*Reduced by Basic Filing Fee Paid			
SUBTOTAL (3) (\$)			

(Complete if applicable)

SUBMITTED BY				
Name (print/type)	RICHARD J. LONG	Registration No.	48252	Telephone 978-687-9200
Signature	<i>Richard J. Long</i>	Date	MAY 25, 2004	

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

This collection of information is required by 37 CFR 1.17 and 1.27. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

PROVISIONAL PATENT
0656-032-US2

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: **Steven J. Saul et al.**
Docket No.: **0656-032-US2**
Title: **Inhibition Assay Method and Device for Detection of Antibiotics**

Mail Stop Provisional Patent Application
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

EXPRESS MAIL CERTIFICATE

"Express Mail" label number: ET311327599US
Date of Deposit: May 25, 2004

I hereby certify that the following attached paper(s) or fee:

PROVISIONAL PATENT APPLICATION COVER SHEET (2 pages)
SPECIFICATION (27 pages)
DRAWING SHEETS (2 pages)
CHECK (\$80.00)
FEE TRANSMITTAL (1 page in duplicate)
POSTCARD

are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date indicated above and are addressed to Mail Stop Provisional Patent Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.


Rachael Rodriguez

Enclosures: Provisional Patent Application Cover Sheet (2 pages)
Specification (27 pages)
Drawing Sheets (2 pages)
Check (\$80.00)
Fee Transmittal (1 page)
Postcard

PROVISIONAL APPLICATION COVER SHEET
Additional Page

PTO/SB/16 (04-04)

Approved for use through 07/31/2006. OMB 0651-0032

U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

Docket Number
0656-032-US2

INVENTOR(S)/APPLICANT(S)

Given Name (first and middle if any)	Family or Surname	Residence (City and either State or Foreign Country)
MARTIN T.	ROSSI	METHUEN, MA
ROBERT S.	SALTER	READING, MA

[Page 2 of 2]

Number 2 of 2

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

INHIBITION ASSAY METHOD AND DEVICE FOR DETECTION OF ANTIBIOTICS

BACKGROUND OF THE INVENTION

Antibiotics used in animal feed or to treat farm animals such as dairy and beef cattle and swine occasionally contaminate the food supply. These antibiotics include, for example, beta-lactams, sulfonamides, macrolides, tetracyclines, amphenicols and macrolides. The hazards associated with these undesirable residues include allergic reactions, propagation of resistant organisms and other long-term health risks. For that reason, government agencies such as USDA and FDA require monitoring of such residues in foods. Foods such as milk and meat are routinely tested for the presence of such residues. It is an object of this invention to provide a user-friendly method for detecting a variety of residues in a single, broad-spectrum test.

Kidneys of slaughtered animals are routinely screened for the presence of antibiotics. Current commercially available detection tests for antibiotics in kidney include the STOP TEST for hog kidney and the FAST TEST for veal bob kidney. The STOP TEST is a 24 hour test and the FAST TEST is a 6 hour test. Although the FAST TEST provides more rapid results, a problem with the FAST TEST, particularly in the pork industry, is over-sensitivity to certain antibiotics. In addition, both the STOP and FAST test are procedurally cumbersome, requiring incubation of a sample swab on a lawn of bacterial culture.

Sensitivity of currently available inhibition tests is based primarily on the growth organism and concentration of growth organism, vessel dimensions, media volume, type of media, mix of nutrients, incubation time and temperature. In most, if not all, cases adjusting test sensitivity using one of those parameters changes test sensitivity to all drugs. If a test is adequately sensitive to some drugs, but hypersensitive to other drugs, other methods must be employed.—A particular commercially available test that is hypersensitive to some drugs is the PREMI Test (PREMI Test is a registered trademark of DSM Netherlands).

The PREMI Test is a relatively rapid test (2.5-4 hours) that detects a broad range of antimicrobial drugs below both the European multi drug residue levels (MRL) and United States safe levels. The PREMI Test also suffers from oversensitivity to certain antimicrobials, particularly the beta-lactam drugs. Another problem with the PREMI Test and other current methods is that they require either a cumbersome extraction procedure, or use of organic solvents to remove the drug to be detected from the tissue. For example, the PREMI Test requires extraction with acetonitrile/acetone (70:30%). The acetonitrile must be removed from the sample prior to test operation. This adds a cumbersome procedural step.

In addition to problems with testing time, over-sensitivity and extraction none of the currently available tests are provided in an all-in-one format in which all test reagents and sampling devices are provided in one test instrument.

SUMMARY OF THE INVENTION

It is an object of this invention to provide an easy-to-use test apparatus and method for the microbial culture, growth inhibition, detection of antibiotics in a test sample. In an embodiment, the method and apparatus includes all of the reagents, premixed together and ready for use. Such a pre-mixture can include, for example, nutrients, agar, spores and color indicator. The user only has to add the sample, or sample extract, and incubate for a prescribed time period, for example about 1.5 to 4 hours, at a prescribed temperature, for example about 55-70 degrees C. After test completion results can be retained at room temperature for an extended period, for example 6-8 hours or more, without a change in color and, therefore, change in test result interpretation.

One embodiment includes at least two buffers, wherein one of said buffers has a pka of above 7, for example about 8 to about 11, and the other of said buffers has a pka of below 7, for example about 4.5 to about 6.5. In an example, the low pka buffer is succinate and the high pka buffer is borate. In another example, the high pka buffer is trizma base. In

another embodiment the test method includes reducing antibiotic sensitivity of a microbial culture by contacting said culture with at least one adjustment binder, for example a microbial enzyme, for at least one antibiotic or other culture growth inhibitor. Examples of such microbial enzymes includes a microbial cell wall, antibiotic receptor isolated from *Bacillus* (*Geobacillus*) *Stearothermophilus* (*B.st*).

Another embodiment of the invention is a test apparatus for the microbial culture, growth inhibition, detection of antibiotics in a test sample. The test apparatus can include an agar matrix within a reagent chamber. The agar matrix generally includes agar, nutrients, at least two buffers and spores. The agar matrix can be provided within a reagent chamber. The reagent chamber can include a sealed bottom end and a membrane seal over the top end. In test operation the user can puncture the seal with, for example, a pipet tip and then dispense the sample, for example a 200 microliter sample of milk, into the reagent chamber. The buffers within the agar matrix will generally include at least one buffer with a pka of above 7, for example about 8 to about 11, and at least one buffer with a pka of below 7, for example about 4.5 to about 6.

In an example, the low pka buffer is succinate and the high pka buffer is borate. In another example, the high pka buffer is trizma base and the low pH buffer is succinate. In another embodiment the test method includes reducing antibiotic sensitivity of the microbial culture using an adjustment binder, for example a microbial enzyme that binds with at least one culture growth inhibitor. Examples of such microbial enzymes includes a microbial cell wall, antibiotic receptor isolated from *B.st*.

In an embodiment requiring extraction, the extraction reagents are provided separately from the other reagents. In an example of a method and apparatus for providing extraction reagents, the extraction reagents are provided in a separate reagent compartment and are delivered to the customer as a single test unit. The seals of the reagent compartments are puncturable membrane seals, for example, metallic foil seals. Puncturing the various seals allows mixing of the reagents within the separate compartments. The user only has to add the extracted sample and incubate for a

prescribed time period, for example about 1.5 to about 4 hours, at a prescribed temperature, for example about 55-70 degrees C.

In an example, the complete test unit contains all of the reagents, other than extraction reagents, premixed together and ready for use in an agar medium and, in addition, a sampling instrument, such as a swab or probe. The probe can be used to puncture the various membrane seals separating extraction reagents from the agar medium. The probe can be used to absorb and apply the sample to the reagents within the test apparatus and can comprise an absorbent material such as an absorbent, fibrous, cotton-like or cotton material. In another example, a sampling instrument, such as a swab or probe in the format of a POCKETSWAB (POCKETSWAB is a registered trademark of Charm Sciences, Inc. Lawrence, MA) is provided. The format of the POCKETSWAB provides the advantage of controlled movement of the swab in a test device that provides physical support for the swab. For example, the swab can be controllably moved into the reagent compartment containing an extraction buffer. The extraction buffer can be, for example, a buffer solution capable of extracting antimicrobial drugs from a kidney sample, for example a bovine or porcine kidney sample. Using the POCKETSWAB device the swab can remain in the extraction buffer for the time desired. In addition to the convenience of delivering the swab to the customer in an all-in-one device, the POCKETSWAB provides the physical support so that the swab will remain within the buffer solution without the user providing external support to the swab while soaking in the extracting buffer. After extraction, the swab is moved longitudinally through the second membrane seal of the reagent compartment allowing the extraction buffer solution containing sample to flow onto the agar medium.

In an embodiment, the agar media includes all of the reagents premixed together and ready for use. Such a pre-mixture can include nutrients, agar, spores, color indicator and, optionally, an adjustment binder. Said adjustment binder can be either combined within the agar medium or applied on top of the agar medium. The user only has to add the sample and incubate for a prescribed period, for example about 1.5 to 4 hours, at a prescribed temperature, for example about 55-70 degrees C. The adjustment binder

reduces – or down-regulates- test sensitivity to selected antibiotics or other culture growth inhibitor. Possible adjustment binders include antibiotic receptors isolated from bacteria, monoclonal and polyclonal antibodies.

In a particular embodiment, the test method is sensitivity reduced to the beta-lactam family of antibiotics. A beta-lactam binder is used to selectively reduce test sensitivity to the beta-lactams. Test sensitivity will be unaffected relative to antibiotics or inhibitors other than beta-lactams. In an embodiment, the beta-lactam binder is from *B.st.* and the spores are also of *B.st.*.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a user-friendly method, device and kit for the detection of a broad range of residues of antibacterial compounds in a sample, for example a sample of agricultural product. The user of the test has only to add the sample to be tested, incubate and observe the results. Said results are stabilized for later observation by a combination buffering system.

The mechanism of detection in the present invention is microbial growth inhibition. Microbes useful in the practice of this invention include, for example, *B.st.*, *B. subtilis*, *Bacillus megaterium*, *S. aureus*, *Ps. aeuginosa*, *E. coli* and generally microbes which can be easily cultured and which exhibit detectable growth inhibition in the presence of antibiotics. Other examples of microbial growth inhibition tests include those described in described in U.S. patent numbers 5,354,663 and 5,489,532, the teachings of which are incorporated herein.

In an embodiment of the method and apparatus of this invention, all of the reagents required for antibiotic detection, other than reagents required for extraction of antibiotics from a solid matrix, are combined and provided to the end-user in an agar medium. If required, extraction reagents are provided separately, for example in a reagent compartment.

In an embodiment for detection of antibiotics in a sample requiring extraction, a reagent, such as an extraction buffer; is used. Providing an extraction buffer that will easily extract antimicrobial drugs from a sample avoids complicated extraction methods or use of organic solvents. In an embodiment, the extraction buffer contains a combination of Trizma Base and Potassium Phosphate Monobasic in water at a pH of approximately 7.5. Said extraction buffer can be provided separately from the agar medium or, preferably, for ease of use, in an all in one test device such as the POCKETSWAB format. After sample extraction into the extraction buffer, there is no need to remove the buffer; the buffer is simply combined with the test reagents for incubation.

The POCKETSWAB format is described in U.S. patent number 6,180,395, which is incorporated herein by this reference. When provided in the POCKETSWAB format, the extraction buffer can be within a reagent compartment. Said reagent compartment can be situated within the device, above the agar medium. The reagent compartment includes membrane seals, for example, puncturable foil seals, on both ends to retain the buffer therein. During testing, the swab is removed and contacted with the sample. The swab is used to puncture the first seal thereby placing it within the reagent compartment in contact with the extraction reagent. After sufficient time is allowed for extraction to occur, the second sealed end of the reagent compartment is punctured allowing the contents to flow onto the agar medium. Spore germination and bacterial growth, or lack thereof, is determined by observation of changes in spore germination and/or bacterial growth indicators within the agar medium.

Possible growth indicators include acid/base indicators that undergo a detectable change in the presence of acidic or basic conditions and oxidation/reduction indicators. For example, indicators that change color in the presence of an acid or base such as to purple/bluish color if the environment is acidic or yellow color if basic or neutral. In the case of bacterial growth, or bacterial growth following germination of spores, acid is produced. Thus, indicators will reflect creation of an increasingly acid environment as bacterial growth proceeds. If bacterial growth or spore germination is inhibited, for

example by antibiotics in the sample, the environment will be less acidic. The color of the acid/base indicator will reflect that relatively low acidity.

In a preferred embodiment, a double buffering system is utilized. For example, a combination of buffers, one with a pka of above 7 and another with a pka of below 7, can be combined in the agar media. Such a double buffering system can be used to both stabilize the reagent system prior to test operation and stabilize results after the testing is complete. In a particular example, borate and succinate are used. Borate helps provide a high pH environment to stabilize the agar media during storage prior to test operation. Succinate helps provide a low pH environment to stabilize the pH of the system after test operation. For example, in a negative sample the pH of the agar media will be reduced as spores germinate and bacteria multiply. After test completion the color of the agar media will reflect the test results. The buffer with pka of below 7, for example succinate, will help stabilize the pH of the now acidic environment, thereby minimizing or preventing further color change. External pressure for pH change, for example decreased temperature as the temperature of the test returns to room temperature, may cause the agar media to become more basic. A buffer with pka below 7 will help stabilize the result even upon return to room temperature.

In another embodiment, the buffer with a pka of below 7, for example succinate, is chosen for particular temperature stability properties. Some buffers are known to be sensitive to temperature changes. In certain embodiments, the herein described test is incubated during test operation at above room temperature. Upon test completion the temperature of the agar media will return to room temperature. Choice of buffers – a buffer for which the change in temperature will have a minimal or no impact – may be important. For example, in a negative test the final pH of the agar media will be acidic. As the agar media cools, however, the pH of the system will tend to rise, creating a more positive result. A temperature stable buffer at below pka 7 will help stabilize this result and allow, for example, for overnight test development with test result observation occurring in the morning. In one embodiment, succinate is used in combination with borate. In another embodiment trizma is used in combination with succinate.

Choice of buffers will be governed by a variety of factors. One factor is avoiding buffers that are particularly sensitive to the temperature changes within the test. Another factor is the starting pH of the particular buffer. For example, trizma base may provide less temperature stability as compared to borate. Trizma base, however, can provide a more basic starting pH and, therefore, possibly better test sensitivity and stability as compared to borate.

One of the benefits to microbial inhibition type tests is that they tend to be broad spectrum tests. For example, both beta-lactam and sulfonamide antibiotics will cause inhibition of germination and growth of *B.st.*. This compares, for example, to family or antibody specific antibody based tests. It may be desirable, however, to reduce – or down-regulate - test sensitivity of a microbial growth inhibition assay to certain antibiotics, or other inhibitors, for which the test is overly sensitive. The sensitivity of the currently available microbial inhibition tests are based in large part on the particular growth organism used, concentration of bacteria or spores, the nutrients provided and the incubation time and temperature used. In an embodiment, a bacterial receptor is utilized as an adjustment binder alone, or in combination with specific adjustment antibodies. Bacterial receptors that are sensitive to multiple antibiotics can allow sensitivity adjustment of multiple antibiotics to which the particular bacterial receptor is sensitive.

Particularly useful bacterial receptor adjustment binders include microbial receptors such as those described in U.S. patents 4,239,745 and 4,239,852, the teachings of which are incorporated herein by this reference. Other possible adjustment binders include antibodies or other receptors such as enzymes capable of binding to the antibiotic requiring down-regulation.

Antibiotic bound by such an adjustment binder is rendered unavailable, or less available, to inhibit the bacterial growth. This unavailability may be the result of, for example in the case of adjustment binders being employed on top of the agar medium, the adjustment binder binding to the antibiotic thereby creating a larger molecule which does not easily

diffuse into the agar. In the case of adjustment binders being combined into the agar medium, or, alternatively when combined into a liquid medium such as a broth, the unavailability may be the result of inactivation or weakening of the antimicrobial properties of the antibiotic through binding with the adjustment binder. The adjustment binders may also be included pre-applied to the sampling swab, for example by covalently binding the adjustment binder to the swab.

Adjustment binders can be either specific to a particular drug for which the unadjusted test is overly sensitive or a binder for multiple drugs. One example of an adjustment binder for multiple drugs is a bacterial receptor, such as the beta-lactam receptor or receptors isolated from *B.st.* such as described in U.S. patents 4,239,745 and 4,239,852. Other possible sources of beta-lactam receptors include *B. subtilis*, *Bacillus megaterium*, *S. aureus*, *Ps. aeuginosa* and *E. coli*. Examples of useful specific adjustment binders for particular drugs include monoclonal or polyclonal antibodies. Examples of use of antibodies for sensitivity adjustment are provided in U.S. Patent 6,319,466 the teachings of which are incorporated herein by this reference.

Bacterial receptors such as those described above can be isolated and purified using known techniques. In an embodiment, the beta-lactam receptor isolated from *B.st.* is used to adjust the sensitivity of a microbial inhibition assay using *B.st.* as the growth organism. In such an embodiment, test sensitivity to all of the beta-lactams is reduced while test sensitivity to other antibiotics to which *B.st.* is sensitive remains unaffected. This is a particular benefit when target sensitivity is European multi-residue levels (MRL's) and/or U.S. safe levels, both of which allow beta-lactam in tissue at above the levels of detection of an unadjusted *B.st.* microbial growth inhibition assay.

Other bacterial receptors that may be useful include antibiotic receptors isolated from various part of bacterial or other cells including the ribosome or part of ribosome to which certain antibiotics bind. Examples of antibiotics that bind to or otherwise inhibit ribosome function include tetracyclines, sulfonamides or fluroquinolones. Bacterial

growth inhibition by antibiotics such as tetracyclines, sulfonamides and fluroquinolones may be adjusted by the addition of such binders.

In one embodiment, agar medium is provided in a reagent chamber such as a vial or vial-like device. Located in the same device can be a reagent compartment containing an appropriate sample preparation, extraction buffer. In an embodiment, an adjustment binder is added to the top of the culture medium. In another embodiment, an adjustment binder is mixed into the culture medium. In still another embodiment, the adjustment binder is stored separately and added to the culture media along with the sample addition, prior to sample addition or soon thereafter.

Although the described embodiments include an agar matrix, it is also possible to adjust test sensitivity of a microbial inhibition assay, using the herein described adjustment binders, in a liquid growth matrix.

Although we describe combining all test reagents, except sample preparation extraction buffer, in an agar medium, it is equally possible, although less user-friendly, to package some of the reagents separately. For example, nutrients can be provided in a separate compartment or in a separate tablet for addition to the agar medium.

In another embodiment multiple test samples can be tested using a test plate for example a 96 well test plate. In such an embodiment media culture and adjustment binders can be provided together in the well.

Description of the Drawings and Attachments

Figures 1A and 1B illustrate the invention in the format of the POCKETSWAB-PLUS type device. Figure 1A is a schematic view of the swab removed from the test device and Figure 1B is a schematic view of the test device containing the swab. In use of the swab type device of the invention, the swab 1 is removed from the body 3, by gripping the swab handle 2, and contacting the swab with the sample. The swab 1 is then reinserted into the body 3 and screwed longitudinally through the covering 9 of the

reagent chamber 4 and through the covering 10 of the reagent compartment 5 and into bottom of the reagent chamber 4. In an embodiment, the reagent compartment 5 contains an extraction buffer and the agar medium 6 is in the bottom portion of the reagent chamber 4. No extraction buffer is required for certain embodiments. In such embodiments, the reagent chamber may be provided without the reagent compartment.

Figures 1C and 1D illustrates use of the invention without the all-in-one format. Figure 1C illustrates the reagent chamber. Sample can be contacted with the agar matrix 6 within the reagent chamber 4 either by breaking through the covering 9 with a pipet tip and pipetting the sample onto the agar or by using the swab to contact and absorb the sample. The swab 1 is then inserted into the reagent chamber 4 and pushed longitudinally through the covering 9 of the reagent chamber 4 and into bottom of the reagent chamber 4.

Figure 1E illustrates four interconnected reagent chambers 20. The reagent chambers are interconnected by a breakable material 21 so that one or more reagent chambers can be utilized. Within the bottom portion of each reagent chamber 4 is the agar medium 6. At the top of the reagent chamber 4 there is a puncturable covering 9 which can be a puncturable membrane or foil seal. The reagent chamber 4 can also be threaded 24 for optional attachment to the PocketSwab test apparatus. In test operation a swab or pipet tip can be used to puncture the seal 9 on top of the reagent chamber 4 prior to application of test sample to the agar medium 6.

Example 1: Preparation of Agar Medium

The following agar medium can be used for detection of antibiotics and other inhibitors in a variety of matrices including, for example, urine, milk or kidney samples.

1) Bromo cresol purple (BCP)/Tris Solution

Prepare Trizma base solution (TBS) by adding 2.5 grams Trizma Base to 100 mL of reverse osmosis/deionized water (RO/DI Water).

Add 100 milligrams of BCP to 25 mL TBS and mix well.

2) Media Preparation

Dissolve 5 grams glucose and 1 gram Mueller Hinton Broth into 100 mL RO/DI Water.

Prepare a 0.01 mg/mL solution of trimethoprim in RO/DI Water and add 1.2 mL to media.

Add 20 mL of BCP/Tris (sterile filtered through 0.45 micron filter).

3) Combine 0.3 grams Difco Bacto-Agar (Item # 0140-01), 0.225 NaCl and 17.485 RO/DI Water. Mix and heat to 95° C. Remove from heat and allow to cool to 75° C. Add 6 mL media (with BCP/Tris) and mix for 5 minutes. Cool to 57°C and add 1 mL spore (concentration of 1 billion cfu/mL) and mix for 5 minutes. Dispense 0.200 mL into the reagent chamber.

Note: To efficiently dispense agar medium, containing spores of *B.st.*, into the bottom of reagent chamber 4, the agar must be heated to approximately 57 degrees C. In an embodiment, dispensing is done rapidly, for example in less than one hour, preferably in 45 minutes or less, so that the agar medium can be quickly cooled. If the agar medium is not quickly cooled, spores of *B.st.* will germinate prematurely. That is, spores will germinate prior to application of the sample. Excess premature germination will reduce test sensitivity.

Another method for preventing premature spore germination, which can be used alone or in conjunction with rapid dispensing, is to increase the pH of the agar medium, for example to pH 9. The increase in pH provides non-optimal conditions for spore germination.

After dispensing, run tests to determine if sensitivity is adequate. If sensitivity is not adequate repeat procedure using either more spores (if undersensitive) or less spores (if oversensitive).

Example 2: Single Service Kidney Test

The dispensed reagent chamber is prepared as described in example 1.

Extraction buffer is prepared as follows:

Prepare extraction buffer containing 47.2% Trizma Base and 52.5% Potassium Phosphate Monobasic. Add 4.8 grams of the mixture to 1000 mL RO/DI Water (pH should be 7.5 +/- .10).

Seal extraction buffer within reagent compartment 5 and add to reagent chamber 4. Heat seal reagent chamber 4 with Marsh heat sealing foil.

Farm Kidney Swab Procedure (Note: this procedure is described below using a format in which the swab is provided packaged within the test unit such as in the PocketSwab.)

1. Make a 3 inch incision into kidney.
2. Withdraw the swab by gently pulling and twisting handle out of swab body.
3. Insert swab into incision of kidney for 15 minutes to allow full absorption of liquid into the swab.
4. Hold the Kidneyswab nearly upright with the microtube pointing down for the remainder of the test.
5. Reinsert the swab by gently pushing down and twisting to engage threads.
6. Screw the handle down slowly about halfway. The swab tip will puncture the seals and immerse the swab into the extraction buffer. Let sit in buffer for 2 minutes.
7. Screw swab all the way. Shake down liquid and tap several times so residual liquid enters vial completely and place into heat block set at 67°C.
8. Incubate for 2.5 hours (if urine is tested instead of kidney, incubate for 4 hours).
9. Remove vials from incubator and observe vial color in comparison to reference colors. If negative control does not match color specified on protocol incubate an additional 10 minutes.

Using the above procedure with the further addition of various amounts of beta-lactam receptor units to the top of the culture media, from 0.3 units to 5.0 units, test sensitivity to penicillin G, was reduced from 5 parts per billion to between 12.5 and 25 parts per billion. See example 4 below for receptor unit determination.

Example 3: Single Service Urine Test

The agar medium is prepared as described in example 1. The extraction buffer is prepared as described in example 2:

Farm Urine Swab Procedure (Note: this procedure is described below using a format in which the swab is provided packaged within the test unit such as in the PocketSwab as shown in Figure 1B.)

1. Insert swab into urine sample for 10 seconds to allow full absorption into the swab.
2. Hold the swab nearly upright with the microtube pointing down for the remainder of the test.
3. Reinsert the swab by gently pushing down and twisting to engage threads.
4. Screw the handle down slowly about halfway. The swab tip will puncture the seals and immerse the swab into the extraction buffer. Let sit in buffer for 2 minutes.
5. Screw swab all the way. Shake down liquid and tap several times so residual liquid enters vial completely and place into heat block set at 67°C.
6. Incubate for 2.5 hours (if urine is tested instead of kidney, incubate for 4 hours).
7. Remove vials from incubator and observe vial color in comparison to reference colors. If negative control does not match color specified on protocol incubate an additional 10 minutes.

Example 4: Sensitivity Adjusted Single Service Kidney Test with Adjustment Binder (Receptor) Combined with Test Reagents

Agar medium is prepared as in Example 1. Prior to dispensing 200 microliters into reagent chamber 4, one inhibitory receptor unit is mixed into the agar medium.

Determination of one receptor unit is made as follows:

Receptor is defined as protein removed from cellular membrane of *B.st.*, ATCC# 10149

Required Chemicals:

- i)50 ml Triton X-100 - Aldrich (catalog # 23,472-9)
- ii)100 grams Sodium phosphate, dibasic, Anhydrous (Na_2HPO_4) - Sigma (catalog # S-9763)
- iii)50 grams Bovine serum albumin (BSA), protease-free Serologicals

(catalog # 3100-01)

iv) 100 grams Trichloroacetic acid (TCA) – Sigma catalog # T-9159

v) 50 grams Sodium hydroxide (NaOH) Sigma (catalog # S-0899)

Required Equipment:

Radioactive waste container; Charm 6600 analyzer; Calibrated pH meter; 4°C refrigerator; Fume hood; 55°C incubator; Eye protection and gloves; Corrosive tape; Graduated cylinders (100 + 500 ml's); Erlenmeyer flask (250 and 500 ml's); Small weighboats; Spatulas; Test tubes; 2-inch stirbars; Stirplate; Vortex; Parafilm; Absorbent towel.

Buffer S formulation

All concentrations below are grams per liter water.

Final pH of Buffer S is pH 8.9

5.55 Trizma Base

2.25 Sodium Chloride (NaCl)

1.0 Potassium Sorbate (C₆H₇O₂K)

1.0 Sodium Benzoate (C₂H₅O₂Na)

0.12 Benzalkonium Chloride

0.105 Potassium Chloride (KCl)

0.08 Calcium Chloride Hydrate (CaCl₂-H₂O)

0.05 Sodium Bicarbonate (CHNaO₃)

Receptor Unit Determination Test Preparation

Buffer Preparation

A. Prepare 500 mL of Buffer S with 0.05% bovine serum albumin (BSA), pH 7.00 as follows.

1. In a clean 500 mL graduated cylinder, measure out 500 mL of Buffer S.
2. Add the measured buffer S to a clean 500 mL Erlenmeyer flask with a 2-inch stirbar.
3. Weigh 0.25 ± 0.01 g of BSA into a clean weigh boat.

4. Transfer the BSA into the flask of Buffer S, then mix on stirplate with medium speed setting until the BSA is fully dissolved.
 5. Using a pH meter calibrated to 4.0, 7.0 and 10.0, adjust the pH of the solution to 7.00 ± 0.05 with 1 M HCl.
 6. Seal flask with parafilm and then store in a 4°C refrigerator.
- B. Prepare 100 mL of 30% Trichloroacetic acid (TCA):
1. Weigh $30.0 \text{ g} \pm .5 \text{ g}$ trichloroacetic acid directly into a clean 250 mL Erlenmeyer flask with a 2-inch stirbar and a mark at 100 mL.
 2. Bring the volume of the TCA solution to the 100 mL mark with ULTRA PURE H_2O . Mix on stirplate with medium speed setting until the TCA is fully dissolved.
 3. Seal flask with parafilm and store at room temperature.
- C. Prepare 250 mL of 0.1% Triton X-100 in 0.2 M NaOH as follows:
1. Weigh $0.25 \pm 0.01\text{g}$ Triton X-100 directly into a clean 500 mL Erlenmeyer flask with mark at 250 ml.
 2. Add 150 mL ULTRA PURE H_2O to the flask of Triton X-100, plus a stir bar, then mix on speed setting 5 until the Triton X-100 has completely dissolved.
 3. Weigh $2.00 \pm 0.1 \text{ g}$ of NaOH (FW = 40.00) in a clean weighing dish. Add the NaOH to the Triton X-100 solution and mix to dissolve.
 4. Bring volume of solution to 250 mL with ULTRA PURE H_2O . Seal flask with parafilm.
 5. Store solution at room temperature.

Procedure

- A. Label a separate test tube for each amount to be tested (10, 20, 30 μL), plus two zeroes.
- B. Add 10 μL , 20 μL , and 30 μL of receptor to the appropriately labeled tube.
- C. bring the volume to 1 mL in each test tube with Buffer (i.e., add 990, 980, and 970 mL, respectively). For the zeroes, use 1 mL Buffer S/BSA solution only.
- D. Add one Charm II 14C penicillin G tracer tablet to each sample, vortex 10 times, then incubate samples at 55°C for 3 minutes.

- E. After 3 minutes, mix samples again by vortexing 5 times, then return samples to 55°C incubator for another 3 minutes.
- F. Add 300 µL of 30% trichloroacetic acid to each sample, then mix samples by vortexing five times.
- G. Add 2 mL ULTRA PURE H₂O to each sample, then centrifuge at 3400 rpm for 5 minutes.
- H. Pour off supernatant from each tube into radioactive waste container, then blot rims dry in absorbent towel.
- I. Add 300 µL of 0.1% Triton X-100 in 0.2 M NaOH to each sample, then vortex to dissolve the pellets. Break up pellets completely.
- J. Add 3 mL Optifluor liquid scintillation fluid to each tube, then vortex 10 times.
- K. Count each tube for 1 minute on Charm 6600 analyzer. Record sample cpm results.
- L. If a cpm result is higher than 3000 cpm, dilute the sample and repeat testing.

One inhibitory unit is defined as the volume of receptor solution needed to give a reading that is 1000 cpm's higher than the average zero cpm. Calculate the number of units per mL.

Example 5: Preparation of Agar Media Containing Two Buffer

The following agar medium can be used for detection of antibiotics and other inhibitors in a variety of matrices including, for example, urine, milk or kidney samples and can be used alone in a test container or in the single service test unit in sensitivity adjustment examples.

1) BCP/Borate/Succinate Solution

a) Add 3.8 grams of Borate and 6 grams Succinate to 100 mL of reverse osmosis/deionized water (RO/DI Water) in 125 mL flask.

b) Add 50 milligrams of BCP to 50 mL conical tube.

Add 25 mL of Borate/Succinate solution to the 50 milligrams BCP and mix.

2) Media Preparation

Dissolve 5 grams glucose and 1 gram Mueller Hinton Broth into 100 mL RO/DI Water.

Prepare a 0.01 mg/mL solution of trimethoprim in RO/DI Water and add 1.2 mL to media.

Add 20 mL of BCP/Borate/Succinate to media.

Sterile filter through 0.45 micron filter. Cool to 4 degrees C.

3) Combine 0.3 grams Difco Bacto-Agar (Item # 0140-01), 0.225 NaCl and 17 mL RO/DI Water. Mix and heat to 95° C. Remove from heat and allow to cool to 75° C.

Add 6 mL media and mix for 5 minutes. Cool to 57°C and add 2 mL spore (concentration of 1 billion cfu/mL) and mix for 5 minutes. Dispense 0.200 mL into the reagent chamber.

Note: To efficiently dispense agar medium, containing spores of *Bst*, into the bottom of reagent chamber 4, the agar must be heated to approximately 57 degrees C. In an embodiment, dispensing is done rapidly, for example in less than one hour, preferably in 45 minutes or less, so that the agar medium can be quickly cooled. If the agar medium is not quickly cooled, spores of *B.st.* will germinate prematurely. That is, spores will germinate prior to application of the sample. Excess premature germination will reduce test sensitivity.

Another method for preventing premature spore germination, which can be used alone or in conjunction with rapid dispensing, is to increase the pH of the agar medium, for example to pH 9. The increase in pH provides non-optimal conditions for spore germination.

After dispensing, run tests to determine if sensitivity is adequate. If sensitivity is not adequate repeat procedure using either more spores (if undersensitive) or less spores (if oversensitive).

The following Table 1 shows results from antibiotic detection tests using agar media described in Example 5. In test operation 200 microliters of various milk sample, spiked with known concentrations of antibiotics, were pipetted into test vials and incubated for the prescribed time (in this test 2 hours 10 minutes) and prescribed temperature (64

degrees C +/- 2 degrees C). Results were recorded immediately after test completion and 16 hours later.

Charm Sciences CowSide Test Sensitivity Testing Results

Table 1

Incubation Time: 2 hours and 10 minutes at 64°C in various Charm Incronic 1 Incubators.

Drug:	Concentration:	Color (Initial):	% Positive	Color (16 hr/RT)	% Positive Tolerance Levels	
					USA	EU/MRL
Pen G	4 ppb	5	100% (4/4)	5	100% (4/4) 5	4
Pen G	3 ppb	5	100% (2/2)	5	100% (2/2) 5	4
Amoxicillin	6 ppb	5	100% (2/2)	5	100% (2/2) 10	4
Ampicillin	5 ppb	5	100% (4/4)	4	100% (4/4) 10	4
Cloxacillin	30 ppb	4	100% (4/4)	5	100% (4/4) 10	30
Cloxacillin	50 ppb	5	100% (4/4)	5	100% (4/4) 10	30
Oxytetracycline	200 ppb	2	0% (0/4)	2	0% (0/4) 300	100
Oxytetracycline	300 ppb	5	100% (4/4)	4	100% (4/4) 300	100
Sulfamethazine	100 ppb	5	100% (4/4)	5	100% (4/4) 10	100
Sulfamethazine	200 ppb	5	100% (4/4)	4	100% (4/4) 10	100
Sulfadimethoxine	25 ppb	4	100% (4/4)	5	100% (4/4) 10	100
Sulfadimethoxine	50 ppb	5	100% (4/4)	5	100% (4/4) 10	100
Gentamicin	300 ppb	4	100% (4/4)	4	100% (4/4) 30	100
Gentamicin	400 ppb	5	100% (4/4)	5	100% (4/4) 30	100
Tylosin	40 ppb	5	100% (4/4)	4	100% (4/4) 50	50
Tylosin	50 ppb	5	100% (4/4)	5	100% (4/4) 50	50
Pirlimycin	100 ppb	4	100% (4/4)	4	100% (4/4) 400	100
Pirlimycin	200 ppb	5	100% (4/4)	5	100% (4/4) 400	100
Ceftiofur Sodium	50 ppb	1	0% (0/4)	1	0% (0/4) 50*	100^
Ceftiofur Sodium	100 ppb	2	0% (0/4)	2	0% (0/4) 50*	100^
Cephapirin	10 ppb	5	100% (4/4)	5	100% (4/4) 20	60
Sulfadoxine	100 ppb	5	100% (4/4)	4	100% (4/4) Not listed	Not listed
Tilmicosin	80 ppb	5	100% (4/4)	5	100% (4/4) Not listed	Not listed
Neomycin	750 ppb	5	100% (4/4)	5	100% (4/4) Not listed	Not listed

* Parent Drug Listed

^ Total parent and metabolites

Raw Milk: 0 1 0% (0/16**) 1 0% (0/16) N/A N/A

** Represents Individual Bulk Tanker Samples

Example 6: Preparation of Agar Medium Containing Two Buffer

The following agar media can be used for detection of antibiotics and other inhibitors in a variety of matrices including, for example, urine, milk or kidney samples and can be used in a variety of formats including single service or sensitivity adjustment examples.

1) BCP/Trizma/Succinate Solution

- a) Add 2.5 grams Trizma base and 6 grams Succinate to 100 mL of reverse osmosis/deionized water (RO/DI Water) in 125 mL flask.
- b) Add 40 milligrams of BCP to 50 mL conical tube.

Add 25 mL of Trizma/Succinate solution to the 40 milligrams BCP and mix.

2) Media Preparation

Dissolve 5 grams glucose and 1 gram Mueller Hinton Broth into 100 mL RO/DI Water.

Prepare a 0.01 mg/mL solution of trimethoprim in RO/DI Water and add 1.2 mL to media.

Add 20 mL of BCP/Trizma/Succinate to media.

Sterile filter through 0.45 micron filter. Cool to 4 degrees C.

3) Combine 0.3 grams Difco Bacto-Agar (Item # 0140-01), 0.225 NaCl and 17 mL RO/DI Water. Mix and heat to 95° C. Remove from heat and allow to cool to 75° C. Add 6 mL media (with BCP/Tris) and mix for 5 minutes. Cool to 57°C and add 2 mL spore (concentration of 1 billion cfu/mL) and mix for 5 minutes. Dispense 0.200 mL into the reagent chamber.

Note: To efficiently dispense agar medium, containing spores of *Bst*, into the bottom of reagent chamber 4, the agar must be heated to approximately 57 degrees C. In an embodiment, dispensing is done rapidly, for example in less than one hour, preferably in 45 minutes or less, so that the agar medium can be quickly cooled. If the agar medium is not quickly cooled, spores of *B.st.* will germinate prematurely. That is, spores will germinate prior to application of the sample. Excess premature germination will reduce test sensitivity.

Another method for preventing premature spore germination, which can be used alone or in conjunction with rapid dispensing, is to increase the pH of the agar medium, for

example to pH 9. The increase in pH provides non-optimal conditions for spore germination.

After dispensing, run tests to determine if sensitivity is adequate. If sensitivity is not adequate repeat procedure using either more spores (if undersensitive) or less spores (if oversensitive).

The following Table 2 shows results from antibiotic detection tests using agar media described in Example 5. In test operation 200 microliters of various milk samples, spiked with known concentrations of antibiotics, were pipetted into test vials and incubated for the prescribed time (in this test 2 hours 30 minutes) and prescribed temperature (64 degrees C +/- 2 degrees C). Results were recorded immediately after test completion and 16 hours later.

Charm Sciences CowSide Test Sensitivity Testing Results Form:

Incubation Time: 2 hours and 30 minutes at 64°C in various Charm Incronic 1 Incubators.
Results:

Drug:	Concentration:	Test Result	% Positive	16hr Result	% Positive	Tolerance Levels	
						USA	EU/MRL
Pen G	4 ppb	Positive	100% (4/4)	Positive	100% (4/4)	5	4
Pen G	3 ppb	Positive	100% (2/2)	Positive	100% (2/2)	5	4
Amoxicillin	6 ppb	Positive	100% (2/2)	Positive	100% (2/2)	10	4
Ampicillin	5 ppb	Positive	100% (4/4)	Positive	100% (4/4)	10	4
Cloxacillin	30 ppb	Positive	100% (4/4)	Positive	100% (4/4)	10	30
Cloxacillin	50 ppb	Positive	100% (4/4)	Positive	100% (4/4)	10	30
Oxytetracycline	200 ppb	Positive	0% (0/4)	Positive	0% (0/4)	300	100
Oxytetracycline	300 ppb	Positive	100% (4/4)	Positive	100% (4/4)	300	100
Sulfamethazine	100 ppb	Positive	100% (4/4)	Positive	100% (4/4)	10	100
Sulfamethazine	200 ppb	Positive	100% (4/4)	Positive	100% (4/4)	10	100
Sulfadimethoxine	25 ppb	Positive	100% (4/4)	Positive	100% (4/4)	10	100
Sulfadimethoxine	50 ppb	Positive	100% (4/4)	Positive	100% (4/4)	10	100
Gentamicin	300 ppb	Positive	100% (4/4)	Positive	100% (4/4)	30	100
Gentamicin	400 ppb	Positive	100% (4/4)	Positive	100% (4/4)	30	100
Tylosin	40 ppb	Positive	100% (4/4)	Positive	100% (4/4)	50	50
Tylosin	50 ppb	Positive	100% (4/4)	Positive	100% (4/4)	50	50
Pirlimycin	100 ppb	Positive	100% (4/4)	Positive	100% (4/4)	400	100
Pirlimycin	200 ppb	Positive	100% (4/4)	Positive	100% (4/4)	400	100
Ceftiofur Sodium	50 ppb	Positive	100% (4/4)	Positive	100% (4/4)	50*	100^
Ceftiofur Sodium	100 ppb	Positive	100% (4/4)	Positive	100% (4/4)	50*	100^
Cephapirin	10 ppb	Positive	100% (4/4)	Positive	100% (4/4)	20	60
Sulfadoxine	100 ppb	Positive	100% (4/4)	Positive	100% (4/4)	Not listed	Not listed
Tilmicosin	80 ppb	Positive	100% (4/4)	Positive	100% (4/4)	Not listed	Not listed
Neomycin	750 ppb	Positive	100% (4/4)	Positive	100% (4/4)	Not listed	Not listed

* Parent Drug Listed

^ Total parent and metabolites

Raw Milk: 0 Negative 0% (0/16**) Negative 0% (0/16) N/A N/A

** Represents Individual Bulk Tanker Samples

Example 7: Preparation of Agar Medium Containing Two Buffer

The following agar medium can be used for detection of antibiotics and other inhibitors in a variety of formats and matrices including, for example, urine, milk or kidney samples.

1) BCP/Borate/Succinate Solution

a) Add 3.8 grams of Borate and 2.7 grams Succinate to 100 mL of reverse osmosis/deionized water (RO/DI Water) in 125 mL flask.

b) Add 100 milligrams of BCP to 50 mL conical tube.

Add 25 mL of Borate/Succinate solution to the 100 milligrams BCP and mix.

2) Media Preparation

Dissolve 5 grams glucose into 100 mL RO/DI Water.

Prepare a 0.01 mg/mL solution of trimethoprim in RO/DI Water and add 1.2 mL to media.

Add 20 mL of BCP/Borate/Succinate to media.

Sterile filter through 0.45 micron filter. Cool to 4 degrees C.

3) Combine 0.3 grams Difco Bacto-Agar (Item # 0140-01), 0.225 NaCl and 18 mL RO/DI Water. Mix and heat to 95° C. Remove from heat and allow to cool to 75° C. Add 6 mL media (with BCP/Tris) and mix for 5 minutes. Cool to 57°C and add 1 mL spore (concentration of 1 billion cfu/mL) and mix for 5 minutes. Dispense 0.200 mL into the reagent chamber.

Note: To efficiently dispense agar medium, containing spores of Bst, into the bottom of reagent chamber 4, the agar must be heated to approximately 57 degrees C. In an embodiment, dispensing is done rapidly, for example in less than one hour, preferably in 45 minutes or less, so that the agar medium can be quickly cooled. If the agar medium is not quickly cooled, spores of Bst will germinate prematurely. That is, spores will germinate prior to application of the sample. Excess premature germination will reduce test sensitivity.

Another method for preventing premature spore germination, which can be used alone or in conjunction with rapid dispensing, is to increase the pH of the agar medium, for

example to pH 9. The increase in pH provides non-optimal conditions for spore germination.

After dispensing, run tests to determine if sensitivity is adequate. If sensitivity is not adequate repeat procedure using either more spores (if undersensitive) or less spores (if oversensitive).

Claims

1. A method for microbial inhibition analyte detection comprising buffering test components with at least two buffers, wherein one of said at least two buffers has a pka of above 7 and the other of said buffers has a pka of below 7.
2. The method of claim 1 wherein one of said at least two buffers has a pka of about 8 to about 11 and the other of said buffers has a pka of about 4.5 to about 6.5.
3. The method of claim 1 wherein one of said buffers is succinate.
4. The method of claim 1 wherein one of said buffers is borate.
5. The method of claim 1 wherein one of said buffers is trizma base.
6. The method of claim 1 further comprising a method for reducing antibiotic sensitivity of a microbial culture comprising contacting said culture with at least one adjustment binder for at least one culture growth inhibitor.
7. The method of claim 1 wherein said at least one adjustment binder comprises a microbial enzyme.
8. The method of claim 7 wherein said at least one adjustment binder comprises a microbial cell wall, antibiotic receptor isolated from *B.st.*.
9. A test apparatus for the microbial culture, growth inhibition, detection of antibiotics in a test sample said test apparatus comprising an agar matrix within a reagent chamber, said agar matrix comprising agar, nutrients, at least two buffers and spores and said reagent chamber comprising a sealed bottom end and a membrane seal over the top end and wherein one of said buffers has a pka of above 7 and the other of said buffers has a pka of below 7.

10. The apparatus of claim 9 further characterized in that one buffer has a pka of about 8 to about 11 and the other of said buffers has a pka of about 5 to about 6.

11. The apparatus of claim 9 further characterized in that the buffer with a pka of from about 8 to about 11 comprises trizma base.

12. The apparatus of claim 9 further characterized in that the buffer with a pka range of from about 8 to about 11 comprises borate.

13. The apparatus of claim 9 further characterized in that the buffer with a pka range of from about 4.5 to about 6.5 comprises succinate.

14. The test apparatus of claim 9 further comprising a method for reducing antibiotic sensitivity of a microbial culture comprising contacting said culture with at least one adjustment binder for at least one culture growth inhibitor.

15. The test apparatus of claim 9 wherein said at least one adjustment binder is combined with said microbial culture.

16. The test apparatus of claim 9 wherein said microbial culture comprises bacterial spores.

17. The test apparatus of claim 9 wherein said microbial culture comprises spores of *B.st.*.

18. A test apparatus for the microbial culture, growth inhibition, detection of antibiotics in a test sample requiring extraction said test apparatus comprising:

- a) at least one container having a membrane seal over a first end and a second end to form a reagent compartment;
- b) an agar matrix within a reagent chamber, said agar matrix comprising agar, nutrients and spores;

c) said reagent chamber comprising a sealed bottom end and a membrane seal over the top end,

wherein, said agar matrix includes at least two buffers and wherein said at least two buffer comprise a buffer at below pKa 7 and a buffer at above pKa 7.

19. The test apparatus of claim 18 wherein an extraction reagent is provided within said reagent compartment.

20. The apparatus of claim 18 wherein said membrane seal comprises a metallic foil seal.

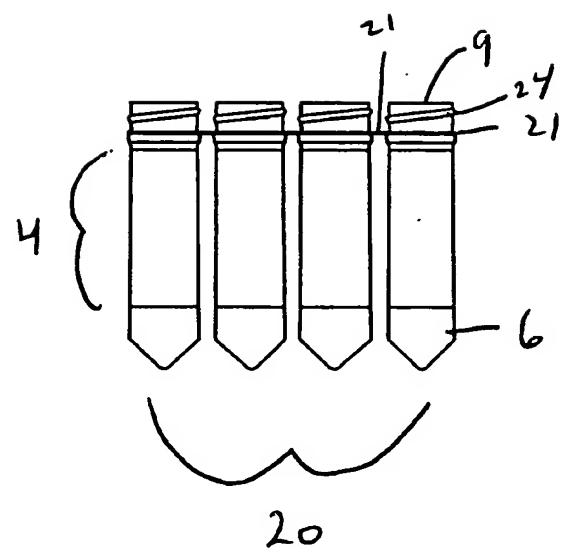
21. The apparatus of claim 18 wherein said spores comprise spores of *Bacillus* (*Geobacillus*)*stearothermophilus*.

22. The test apparatus of claim 18 wherein the growth inhibition of said microbial culture is reduced by the addition of at least one adjustment binder for one or more inhibitors to which said culture is sensitive.

23. The test apparatus of claim 22 wherein said at least one adjustment binder is provided within said reagent compartment.

24. The test apparatus of claim 22 wherein said at least one adjustment binder is provided within said agar matrix.

25. The test apparatus of claim 22 wherein said at least one adjustment binder is added to the top of the agar matrix.



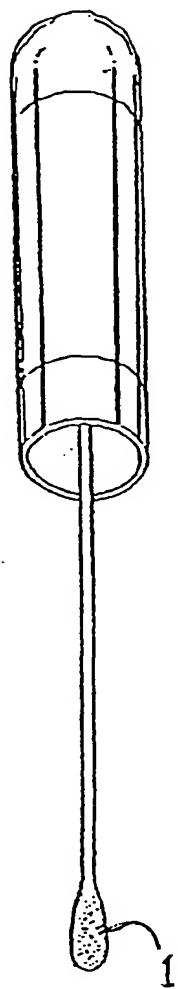


Fig. 1A

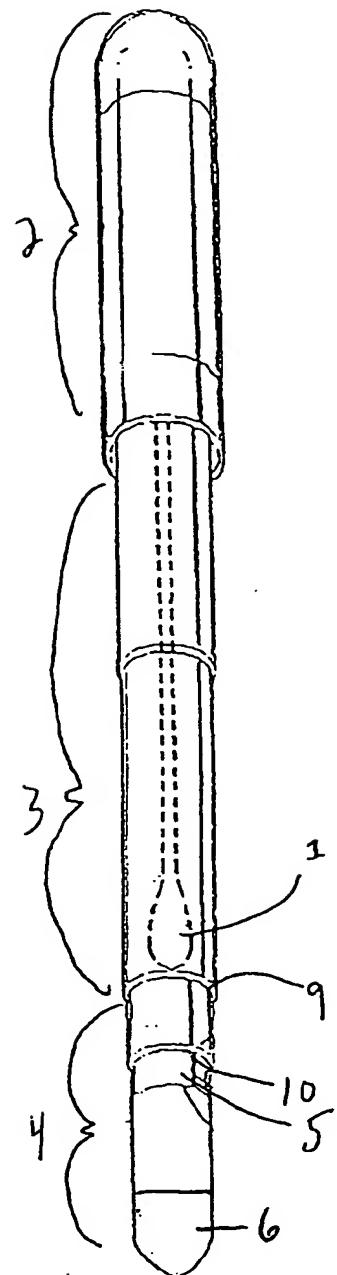


Fig. 1B

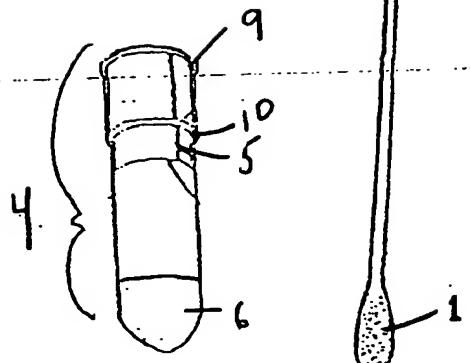


Fig. 1C

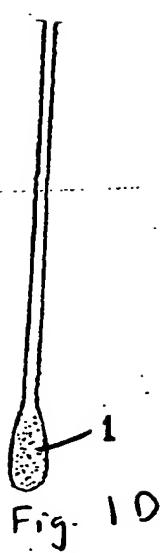


Fig. 1D

Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US04/037220

International filing date: 05 November 2004 (05.11.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US
Number: 60/574,252
Filing date: 25 May 2004 (25.05.2004)

Date of receipt at the International Bureau: 20 December 2004 (20.12.2004)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)



World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record.**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

 BLACK BORDERS

- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT OR DRAWING
- BLURRED OR ILLEGIBLE TEXT OR DRAWING
- SKEWED/SLANTED IMAGES
- COLOR OR BLACK AND WHITE PHOTOGRAPHS
- GRAY SCALE DOCUMENTS

 LINES OR MARKS ON ORIGINAL DOCUMENT

 REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY

- OTHER: _____
-

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.